De Novo Sequencing of Proteolytic Peptides by a Combination of C-Terminal Derivatization and Nano-Electrospray/ Collision-Induced Dissociation Mass Spectrometry

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A series of synthetic peptides (3–15 residues), C-terminally derivatized with 4-aminonaphthalenesulfonic acid (ansa), have been analyzed on a hybrid magnetic sector–orthogonal acceleration time-of-flight tandem mass spectrometer, fitted with a nano-electrospray (nano-ES) interface. Deprotonated molecules generated by negative-ion ES were subjected to collision-induced dissociation (CID) using either methane or xenon as the collision gas, at a collision energy of 400 eV (laboratory frame of reference). As a consequence of charge localization on the sulfonate group, only C-terminal fragment ions were formed, presumably by charge-remote fragmentation mechanisms. Interpretable CID spectra were obtained from fmol amounts of the small peptides (up to 6 residues), whereas low pmol amounts were required for the larger peptides. CID spectra were also recorded of derivatized, previously noncharacterised peptides obtained by proteolysis of cytosolic hamster liver aldehyde dehydrogenase. Interpretation of these CID spectra was based on rules established for the fragmentation of the synthetic peptides. This study shows that derivatization with ansa may be useful in the de novo sequencing of peptides. (J Am Soc Mass Spectrom 2000, 11, 673–686) © 2000 American Society for Mass Spectrometry

With the advent of "soft ionization" techniques [1–5] and commercial tandem mass spectrometers [6–8], peptide sequence analysis by mass spectrometry has become viable. In particular, low flow-rate electrospray (ES) [9–11] has improved sensitivity so that sub-pmol amounts of peptide are now routinely sequenced by tandem mass spectrometry (MS/MS). Although data obtained is often of high quality, its interpretation can be problematic. With the introduction of sequence-tag database search routines [12, 13] peptide sequence analysis by MS/MS can be automated with computer-assisted spectral interpretation. However, peptides obtained from proteins not yet in the databases cannot be identified by this method, and manual interpretation of MS/MS spectra is the necessary alternative. One possible method to simplify the interpretation of MS/MS spectra is to derivatize peptides so as to encourage a particular type of fragmentation. This is not a novel idea. Mass spectrometry groups, particularly those at MIT [14, 15], Michigan State University [16–20], the Imperial Cancer Research Fund [21], Genentech [22], and Karolinska Institutet [23–26], have over the last 20 years been attempting to find a suitable derivatization method to employ in combination with "soft ionization" and MS/MS.

The requirements for an optimal derivative are as follows:

1. The derivatized peptide should fragment in a controlled manner.
2. The derivatization should be quantitative, and applicable to sub-pmol amounts of sample.
3. The derivatization should give a minimum of side products.
4. The experimental procedure should be simple, rapid, and require a minimum of product clean up.

A derivatization method to meet all of the above requirements is not yet available. The strengths and weaknesses of various derivatization methods have recently been discussed in an excellent review by Watson and co-workers [18]. To date most derivatiza-

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tion methods have been designed to couple a positively charged group, e.g., a quaternary ammonium or phosphonium group, to the N-terminus of a peptide so as to promote charge-remote fragmentation (CRF) upon high energy collision-induced dissociation (CID) or matrix-assisted laser desorption/ionization (MALDI) post-source decay (PSD) [14–22]. In this laboratory we have applied a somewhat different approach, in that we have attempted to optimize a procedure to couple aminosulfonic acids to the C-terminus of peptides [23–26]. The resulting sulfonated peptides give abundant deprotonated molecules by negative-ion ES, and fragment in CID reactions in a controlled manner to give a series of CRF ions. In the present report, we describe guidelines for the interpretation of CID spectra of naphthalenesulfonated peptides. We also demonstrate the value of naphthalenesulfonation in combination with negative-ion ES and MS/MS for the sequence analysis of peptides derived from proteolytic cleavages of cytosolic aldehyde dehydrogenase (ALDH) from the liver of the Syrian golden hamster.

Experimental

Materials

The synthetic peptides, amyloid β-protein fragment 31–35 (IIGLM), VGVAPG, β-casomorphin (YPFPGPI), α_1-mating factor fragment 1–6 (WHWLQL), VG-GYGYGAK, bradykinin (RPPGFSPFR), α-neoendorphin (YGGFLRKYPK), PLSRTLSVAAKK, syntide 2 (PLARTLSVAGLPGKK), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and acetic anhydride were from Sigma (St Louis, MO). The peptide LAL was synthesized in this laboratory. 4-Aminonaphthalenesulfonic acid was from Fluka (Buchs, Switzerland). Bovine chymotrypsin was from Merck (Darmstadt, Germany) and *Achromobacter* lysine-specific protease was from Wako Chemicals (Neuss, Germany). Water was deionized and purified with a Milli-Q cartridge system (Millipore, Bedford, MA). Organic solvents were of HPLC grade and distilled prior to use. Gold coated borosilicate capillaries were from Protana A/S (Odense, Denmark).

Methods

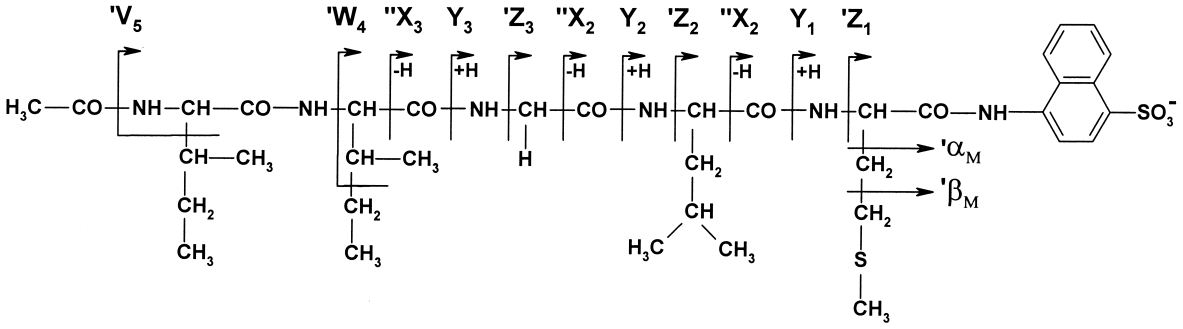
Proteolytic cleavage. Peptides were generated by proteolytic cleavages of cytosolic ALDH purified from livers of Syrian golden hamsters [27]. Separate batches of the carboxymethylated [28] protein were digested at 37 °C for 4 h with bovine chymotrypsin or *Achromobacter* lysine-specific protease in 0.1 M ammonium bicarbonate, pH 8, with 1.8 M urea to aid solubilization. The peptides obtained were purified by reversed-phase high-performance liquid chromatography (HPLC) on a C18 column (Ultropak TSK ODS-120T, 5 μm, 4.6 $\times$ 250 mm, Amersham Pharmacia Biotech, Uppsala, Sweden) using a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid (TFA) (0%–60% in 90 min) in aqueous 0.1% TFA. Fractions were collected and aliquots analyzed by positive-ion nano-ES. Solvent was removed by a stream of nitrogen and the residue stored under vacuum overnight. The peptides were then derivatized.

Derivatization of Peptides. All peptides were acetylated before derivatization with 4-aminonaphthalenesulfonic acid (ansa). A solution of acetic anhydride in methanol, (1:3 v:v, 250 μL) was added to a solution of the peptide (1–100 nmol) in 50 μL of water. After 3 h at ambient temperature, solvents were removed under vacuum and the acetylated peptides were used for derivatization with ansa. When peptides were acetylated on the pmol level, i.e., those obtained from the proteolytic cleavages, the above volumes were scaled down by a factor of 5.

For derivatization with ansa, the acetylated peptides were dissolved in 50 μL of pyridine/HCl coupling buffer, pH 5. The latter was prepared by the addition of 5.3 mL of 32% HCl to a solution of 8 mL of pyridine in 80 mL of water and by the addition of water to make a final volume of 100 mL. 50 μL of 1.0 M EDC and 400 μL of 250 mM ansa, both of which were dissolved in the coupling buffer, were added to the peptide solution. After 2 h at 25 °C the reaction was quenched by the addition of 50 μL of acetic acid. After centrifugation, the solution was injected onto a reversed-phase (C18) HPLC column (Vydac, 5 μm, 4.6 $\times$ 250 mm, Hesperia, CA). Separation of the peptide derivatives from excess EDC, ansa, and coupling buffer was achieved using a linear gradient of acetonitrile containing 0.085% TFA (0%–50% in 50 min) in aqueous 0.1% TFA. The derivatives were collected and solvent removed under a stream of nitrogen. The derivatives were left under vacuum overnight to remove any remaining TFA. In certain instances where abundant Na–TFA adducts were evident in the ES mass spectra, subsequent removal of TFA was achieved by twice adding 100 μL of 10 mM HCl to the peptides and leaving them under vacuum overnight. When derivatizing peptides on the pmol level, as for the peptides obtained from the proteolytic cleavages, the above volumes were scaled down by a factor of 10.

Mass Spectrometry. Positive-ion mass spectra and negative-ion mass and CID spectra were recorded on a Micromass AutoSpec-OATOFFPD hybrid double focusing–orthogonal acceleration time-of-flight (OATOF) tandem mass spectrometer, fitted with a focal plane detector (FPD) after the magnetic sector [29] (Micromass, Manchester, UK). The instrument was operated at an accelerating potential of 4 kV. Peptides dissolved in 50% aqueous methanol were electrosprayed from gold coated borosilicate capillaries with a spraying orifice of about 5 μm (i.d.). 1–3 μL of sample tended to spray for 1–2 h.

Negative-ion CID spectra were recorded using the



Scheme 1. Nomenclature for peptide fragment ions exemplified with the peptide Ac-IIGLM-ansa. For clarity, only some of the cleavages are indicated.

OATOF analyzer. Monoisotopic deprotonated molecules were selected by the double focusing EBE (E = electric, B = magnetic) sectors of the instrument (resolution 2000, 10% valley definition) and transmitted to the OATOF collision cell located just after the final collector slit, and containing methane or xenon as the collision gas. The cell was floated to −3.6 kV thereby giving a collision energy of 400 eV in the laboratory frame of reference. The collision gas pressure was optimized so as to obtain spectra containing a maximum of structural information. This corresponded to an attenuation of the precursor ion beam by 75%–90%. In general, the degree of attenuation required was greatest with methane as the collision gas. Undissociated precursor and product ions were pulsed into the OATOF mass analyzer. Product ion resolution in the resulting spectra was in excess of 1000 (full width at half maximum height definition).

Results and Discussion

Derivatization

The efficiency of the derivatization procedure when applied to synthetic peptides has been discussed elsewhere [23, 26]. The acetylation reaction gives quantitative yields (>95% conversion) in all cases. Both N-terminal and side-chain amino groups become *N*-acetylated. For some of the peptides, methylation of the carboxylic acid groups occurred to a minor extent. The acetylated peptides were quantitatively derivatized with ansa (>95% conversion). The extent of conversion was estimated from the absorption at 206 nm measured during the HPLC separations (the molar absorption of the derivatized and underivatized peptides was found to be similar).

CID of Derivatized Peptides

The nomenclature used to describe peptide fragmentation is from Roepstorff and Fohlman [30] (Scheme **1**). The number of primes to the left of a letter signifying a bond cleavage indicates that the ion formed is deficient in that number of hydrogens as compared to a fragment ion formed by such a homolytic bond cleavage in the molecular ion, e.g., ′X and ″Y. This nomenclature has also been applied to the ′V and ′W fragment ions not originally described by Roepstorff and Fohlman. As there is no commonly accepted nomenclature to describe noncomplete side chains losses, we propose the use of α to describe the cleavage of the bond between the α carbon and the first atom in the side chain. Cleavage of the next bond in the side chain is designated by β. In this manner cleavage of successive bonds in the side chain are designated by successive Greek letters. Primes are used as above.

In an effort to define rules for the interpretation of CID spectra obtained from peptides C-terminally derivatized with ansa, a series of synthetic peptides ranging in size from 3 to 15 residues was derivatized and subjected to CID. The derivatized peptides are listed in Tables 1 and 2 together with the number of amino acid residues that gave at least one sequence specific fragment ion. For most of the peptides, sequence ions from each residue were observed. When the coverage was not complete, the missing fragment ions were always those of highest mass. Medzihradszky et al. [31] have noted this effect in studies on an instrument of similar configuration. The degree of sequence coverage was similar whether methane or xenon was used as the collision gas, but the appearance of the spectra differed.

The amount of sample required for the generation of CID spectra in which most of the sequence ions were present was determined. In general, sensitivity was independent of whether methane or xenon was used as the collision gas. For the four smallest peptides (Tables 1 and 2) fmol amounts were sufficient to generate interpretable spectra, whereas for the larger peptides low pmol amounts were required. The number of amino acid residues in the derivatized peptide is the most important factor in determining sensitivity. For larger derivatized peptides the abundance of the deprotonated molecule is reduced as peptide size increases. This is believed to be a consequence of the increased difficulty in formation of deprotonated molecules as the number of basic sites in the molecule increases.

Fragment ions observed with methane and xenon as

Table 1. Fragment ions observed in the CID spectra obtained with methane as the collision gas. The most abundant fragment ions are shown in boldface. Low-mass fragment ions derived from the ansa residue, fragmentations of the N-terminal acetylamino group, and neutral losses of less than 20 Da from the deprotonated molecules are nondiagnostic and are not included in the table

Peptide sequence	$[M - H]^-$ *m/z*	No. of residues: identified/ total[b]	″X	′X	″Y[e]	Y	Y − 80	″Z	′Z	′V[f] Present	′V[f] Absent	′W[f] Present	′W[f] Absent	′α[f,g] Present	′α[f,g] Absent	′β[f] Present	′β[f] Absent	Other ions[h]
Ac-LAL-ansa	561.2	3/3	1,2	—	1 (AL) 2 (LA)	**1,2**	**1,2**	1	1,2	L,A,**L**	—	L,**L**	—	—	L	—	L	—
Ac-IIGLM-ansa	791.3	5/5	1,2,3	—	2 (GL)	**1,2,3**, 4	**1,2**,3, 4	1	1	I,I,L, M	—	I,**L**,M	I	—	I,L,M	—	I,L,M	—
Ac-VGVAPG-ansa	744.3	6/6	1	—	**2** (AP)	**1,2,3**, 4,5	**1,2**	1	—	—	V,V, A,P	V,P	V	—	V,A	—	—	—
Ac-YPFPGPI-ansa	1035.4	7/7	1,3,5	—	**2** (GP) 3 (PG) **4** (FP) 5 (PF) **6** (YP)	**1,2**,3, **4**,5,**6**	**1,2**,4	1	—	Y,F,I	P,P,P	P,**P**,P, I	Y,F	—	Y,F,I	—	Y,F,I	—
Ac-WHWLQL-ansa	1127.5	6/6	1,2,3, 4	—	1 (QL) 2 (LQ) 3 (WL) 4 (HW) 5 (WH)	**1,2,3, 4,5**	**1,2,3, 4,5**	**1**,2,3, 4,5	1,2	W,H, **W**,L, Q,L	—	**L**,Q,**L**	W,H, W	W	H,L,Q	—	W,H,L, Q	
Ac-VGGYGYGAK[a]-ansa	1158.4	9/9	1,3	—	2 (GA) 3 (YG) 4 (GY)	**1,2,3, 4,5**,6, 7,8	**1**,2,3, 4,5	1,4	—	V,Y, **Y**,K[a]	A	K[a]	V,Y,Y	—	V,Y,A, K[a]	—	Y,K[a]	—
Ac-RPPGFSPFR-ansa	1305.6	9/9[c]	2	—	3 (SP)	**1**,2,**3**, 4,8	**1**,2,3	1,3	—	—	R,P,P, F,S,P, F,R	—	R,P,P, F,S,P, F,R	**S**	R,F	—	R,F	$Y_4\alpha S'$ $Y_5\alpha S'$ $Y_6\alpha S'$ $Y_7\alpha S'$ $Y_8\alpha S'$
Ac-YGGFLRK[a]YPK[a]-ansa	1557.7	10/10	1,4	—	1 (PK[a]) **2** (YP)	**1,2,3, 4,5,6**, 7,8,9	1,**2**	1,3,6	—	Y,Y	F,L,R, K[a],P, K[a]	P	Y,F,L, R,K[a],Y, K[a]	—	Y,F,L, R,K[a]	—	Y,F,L, R,K[a]	—
Ac-PLSRTLSVAAK[a]K[a]-ansa	1599.8	11/12[d]	5,8	5,8	3 (AA)	**1,2,3, 4,5,6, 7**,8,9, 10	**1**,2,3, 4,5	—	—	—	P,L,S, R,T,L, S,V,A, A,K[a],K[a]	L,V	P,L,S, R,T,S, K[a],K[a]	**S,T**	L,R,V, A,K[a]	—	L,R,K[a]	$Y_6\alpha S'$ $Y_7\alpha S'$ $Y_8\alpha S'$ $Y_9\alpha S'$ $Y_{10}\alpha T'$ $Y_{12}\alpha T'$ $\alpha S\ \alpha T'$ $Y_8\alpha T''$ $Y_9\alpha S\alpha T''$ $Y_{12}\alpha S\alpha T''$

(*continued*)

Table 1. (*continued*)

Peptide sequence	$[M - H]^-$ *m/z*	No. of residues: identified/ total[b]	″X	′X	″Y[e]	Y	Y − 80	″Z	′Z	′V[f] Present	′V[f] Absent	′W[f] Present	′W[f] Absent	$'_{\alpha}$[f,g] Present	$'_{\alpha}$[f,g] Absent	$'_{\beta}$[f] Present	$'_{\beta}$[f] Absent	Other ions[h]
Ac-PLARTLSVAGLPGK[a]K[a]-ansa	1837.0	14/15[d]	3,10, 11,12	10,12	4 (LP)	**1**,**2**,3, **4**,5,**6**, **7**,**8**,**9**, **10**, 11, 12,13	**1**,2,4	—	—	L,K[a]	P,A,R, T,L,S, V,A,L, P,K[a]	L,L,P, K[a]	P,R,T, S,V,L, K[a]	**T,S**	L,A,R, V,K[a]	—	L,R, K[a]	$Y_g\alpha S'$ $Y_{10}\alpha S'$ $Y_{11}\alpha T'$ $Y_{12}\alpha T'$ $\alpha S\alpha T'$ $\alpha S\alpha T' - 80$

[a]Lysine residues are ε-*N*-acetylated.
[b]The number of amino acid residues that give at least one sequence ion/the total number of amino acid residues.
[c]Three of the Y fragment ions are absent but $Y\alpha S'$ fragment ions (i.e., Y ions which have lost the side chain of serine) are present. These fragment ions are usually abundant when methane is used as the collision gas.
[d]When sequence ions of all the amino acid residues are not present, those absent are always of highest mass.
[e]The residue on either side of the cleaved amide bond is shown in parentheses.
[f]Because these fragmentations involve cleavage of the amino acid side chain, one-letter amino acid abbreviations are used to indicate where fragmentation occurs.
[g]Serine, threonine, and tryptophan give α instead of $'\alpha$ fragment ions. Discussed in text.
[h]These ions are formed by cleavage of one backbone bond combined with loss of at least one side chain, or by loss of several side chains.

the collision gases are summarized in Tables 1 and 2, respectively.

The following features are common to spectra obtained with *both* collision gases:

- The abundance of product ions is greatest at low mass, and decreases as the mass of the fragment ion increases. This is true for fragment ions of all types (cf. [31]).
- Abundant ″Y fragment ions are observed N-terminal to proline residues (this observation was also made in [23]). Weak ″Y fragment ions are sometimes generated by cleavage of other amide bonds. Abundant fragment ions of this type have been observed by others [16] for peptides C-terminally derivatized with a positively charged group, and in tryptic peptides when cleavage is N-terminal to proline [32].
- Loss of the sulfonic acid moiety is observed from Y fragment ions containing up to five residues, giving Y_n-80 fragment ions. The loss of 80 Da is observed only from Y fragment ions.

The following features are characteristic of spectra obtained with *methane* as collision gas:

- The spectra are dominated by Y fragment ions.
- ′V and ′W fragment ions [33, 34] of medium intensity are frequently observed from derivatized peptides of low mass but become less common as the mass of the peptide increases.
- ″X and ″Z fragment ions are observed occasionally, while ′X and ′Z fragment ions are observed less frequently. The abundance of all of these fragment ions are in most cases low.
- The loss of part or all of an amino acid side chain giving $'\beta$x and $'\alpha$x fragment ions, where X is the amino acid residue (see Scheme **1**), is not observed at optimal collision gas pressures. However, at lower gas pressures these fragment ions are sometimes observed.
- As reported by others [35], peptides containing serine, threonine, and tryptophan give rise to α rather than $'\alpha$ fragment ions. Very abundant αS or αT fragment ions are present if the peptide contains serine or threonine. If more than one hydroxyl-containing amino acid residue is present in the peptide, fragment ions which have lost two or more side chains are observed, e.g., $\alpha S\alpha T'$ fragment ions. The αW fragment ions are normally of low abundance.
- The losses of the side chains of serine and threonine occur in combination with amide bond cleavage giving, for example $Y_n\alpha S'$, $Y_n\alpha T'$ or $Y_n\alpha S\alpha T''$ fragment ions. These ions are observed as satellite peaks 30, 44, or 74 Da below the main fragment ion peak, for serine, threonine, or serine plus threonine, respectively. The αW fragmentation can also occur in combination with other cleavages.

Table 2. Fragment ions observed in the CID spectra obtained with xenon as the collision gas. The most abundant fragment ions are shown in boldface. Low-mass fragment ions derived from the ansa residue, fragmentations of the N-terminal acetylamino group, and neutral losses of less than 20 Da from the deprotonated molecules are nondiagnostic and are not included in the table

Peptide sequence	$[M - H]^-$ *m/z*	No. of residues: identified/ total[b]	″X	′X	″Y[d]	Y	Y − 80	″Z	′Z	′V[e] Present	′V[e] Absent	′W[e] Present	′W[e] Absent	′α[e,g] Present	′α[e,g] Absent	′β[e] Present	′β[e] Absent	Other ions[h]
Ac-LAL-ansa	561.2	3/3	1,2	1,2	2 (LA)	**1**,2	**1**,2	1	1,2	L,A,**L**	—	L,**L**	—	**L**	—	**L**	—	—
Ac-IIGLM-ansa	791.3	5/5	1,2,3	1,3,4	2 (GL) 3 (IG)	**1**,2,3	1,2	1,2	**1**,2,3, 4	I,**I**,L, **M**	—	I[f],**I**,**L**, **M**	I[f]	**I**,**L**,**M**	—	I,L,M	—	—
Ac-VGVAPG-ansa	744.3	6/6	**1**,2,3, 4	1,2,3, 4,5	1 (PG) **2** (AP) 3 (VA) 4 (GV) 5 (VG)	1,**2**,3, 4,5	**1**,**2**,3	**1**	**1**,**3**,4, 5	V,**V**, **A**	P	V,V,**P**	—	**V**	—	—	—	—
Ac-YPFPGPI-ansa	1035.4	7/7	1,2,3, 5	1,2,3, 4,5,6	**2** (GP) 4 (GY) 6 (YP)	1,**2**,3, 4,6	**1**,**2**	1	1,2,3, **5**	Y,F,**I**	P,P,P	P,F,**P**, **P**,**I**	Y	Y,F,I	—	F,I	Y	—
Ac-WHWLQL-ansa	1127.5	6/6	1,2,3	—	2 (LQ)	**1**,**2**,3	**1**	1,2	1,2,3, 4	W,H, **W**,**L**, **Q**,**L**	—	**L**,**Q**,**L**	W,H, W	**W**,H,**L**, Q	—	L	W,H,Q	—
Ac-VGGYGYGAK[a]-ansa	1158.4	8/9[c]	1,2,5	6	1 (AK[a]) **2** (GA) 3 (YG) 4 (GY) 6 (GY)	**1**,**2**,**3**, 4,5,6, 7	**1**,2,5	1,4,6	1,2,3, 4,5	Y,**Y**,A, **K**[a]	V	**K**[a]	V,Y,Y	**V**,Y,K[a]	—	Y,K[a]	—	—
Ac-RPPGFSPFR-ansa	1305.6	9/9	1,5,6, 7	1,2,5, 6,7,8	2 (PF) 3 (SP) 5 (GF) 7 (PP) 8 (RP) 8 (RP)	**1**,2,**3**, 4,5,6, 7	1,2,**3**	**1**,2,3, 4,5	**1**,2,3, 4,5,6	F,S,**F**, **R**	R,P,P, P	P,P,S, **P**,F,**R**	R,F	R,F,S	—	F	R	$Y_4\alpha s'$ $Y_5\alpha s'$ $'Y_5\alpha s$ $'Y_7\alpha s$ $'Y_8\alpha s$ $Y_8\alpha s'$ $'X_6\alpha s$ $V_5\alpha s$ $W_7\alpha s$ $W_8\alpha s$
Ac-YGGFLRK[a]YPK[a]-ansa	1557.7	8/10[c]	**1**,2,3, 4,6	2	**2** (YP)	**1**,**2**,**3**, 4,5,6, 7	**1**,**2**	**1**,3	**1**,3	L,R,K[a], **Y**,**K**[a]	Y,F,P	L,R,K[a], **P**,**K**[a]	Y,F,Y	Y,F,L, K[a]	R	—	Y,F,L, R,K[a]	—

(*continued*)

Table 2. (*continued*)

Peptide sequence	$[M - H]^-$ *m/z*	No. of residues: identified/ total[b]	″X	′X	″Y[d]	Y	Y − 80	″Z	′Z	′V[e] Present	′V[e] Absent	′W[e] Present	′W[e] Absent	′α[e,g] Present	′α[e,g] Absent	′β[e] Present	′β[e] Absent	Other ions[h]
Ac-PLSRTLSVAAK[a]K[a]-ansa	1599.8	12/12	1,2,3, 4,5,6, 7,8	6,7,8	2 (AK[a]) **3** (AA) 4 (VA)	**1,2,3, 4,5**,6, 7,8,9	**1**,2,3, 4,5	**1**,2,3, 4,5,6, 7,8, 10	**1**,2,3, **4**,5,6, 7,8,9, 10,11	L,S,R, T,L,S, V,**A**,A, K[a],**K**[a]	P	L,S,R, T,L,S, V,K[a], **K**[a]	P	L,S,R, T,**V**,K[a]	—	**L**,R,K[a]	—	$Y_6\alpha S'$ $Y_7\alpha S'$ $Y_9\alpha T''$ $Y_9\alpha S\alpha t''$ $W_7\alpha S$ $W_{11}\alpha T$ $\alpha S\alpha T'$
Ac-PLARTLSVAGLPGK[a]K[a]-ansa	1837.0	15/15	1,2,3, 4,5,6, 7,8,9, 11	2,4,5, 8,9, 10,11, 12,14	2 (GK[a]) **4** (LP) 5 (GL) 7 (VA) 12 (AR)	**1,2**,3, **4,5,6, 7,8,9, 10**,11, 12	**1,2**,3, 4	2,9, 10,11	**1**,2,3, 5,6,7, 8,9,10, 11,12, 13,14	L,A,R, T,L,S, V,A,L, K[a],**K**[a]	P,P	P,L,R, T,L,S, V,**L,P,** **K**[a],**K**[a]	—	L,R,T, S,**V**,K[a]	—	**L**,R,K[a]	—	$Y_9\alpha S'$ $Y_{10}\alpha S'$ $Y_{11}\alpha T'$ $Y_{12}\alpha T'$ $W_{14}\alpha T$

[a]Lysine residues are ε-*N*-acetylated.
[b]The number of amino acid residues that give at least one sequence ion/the total number of amino acid residues.
[c]When sequence ions of all the amino acid residues are not present, those absent are always of highest mass.
[d]The residue on each side of the cleaved amide bond is shown in parentheses.
[e]Because these fragmentations involve cleavage of the amino acid side chain, one-letter amino acid abbreviatons are used to indicated where fragmentation occurs.
[f]$'W_{5a}$ is present, $'W_{5b}$ is absent.
[g]Serine and threonine give α instead of ′α fragment ions. Discussed in text.
[h]These ions are formed by cleavage of one backbone bond combined with loss of at least one side chain, or by loss of several side chains.

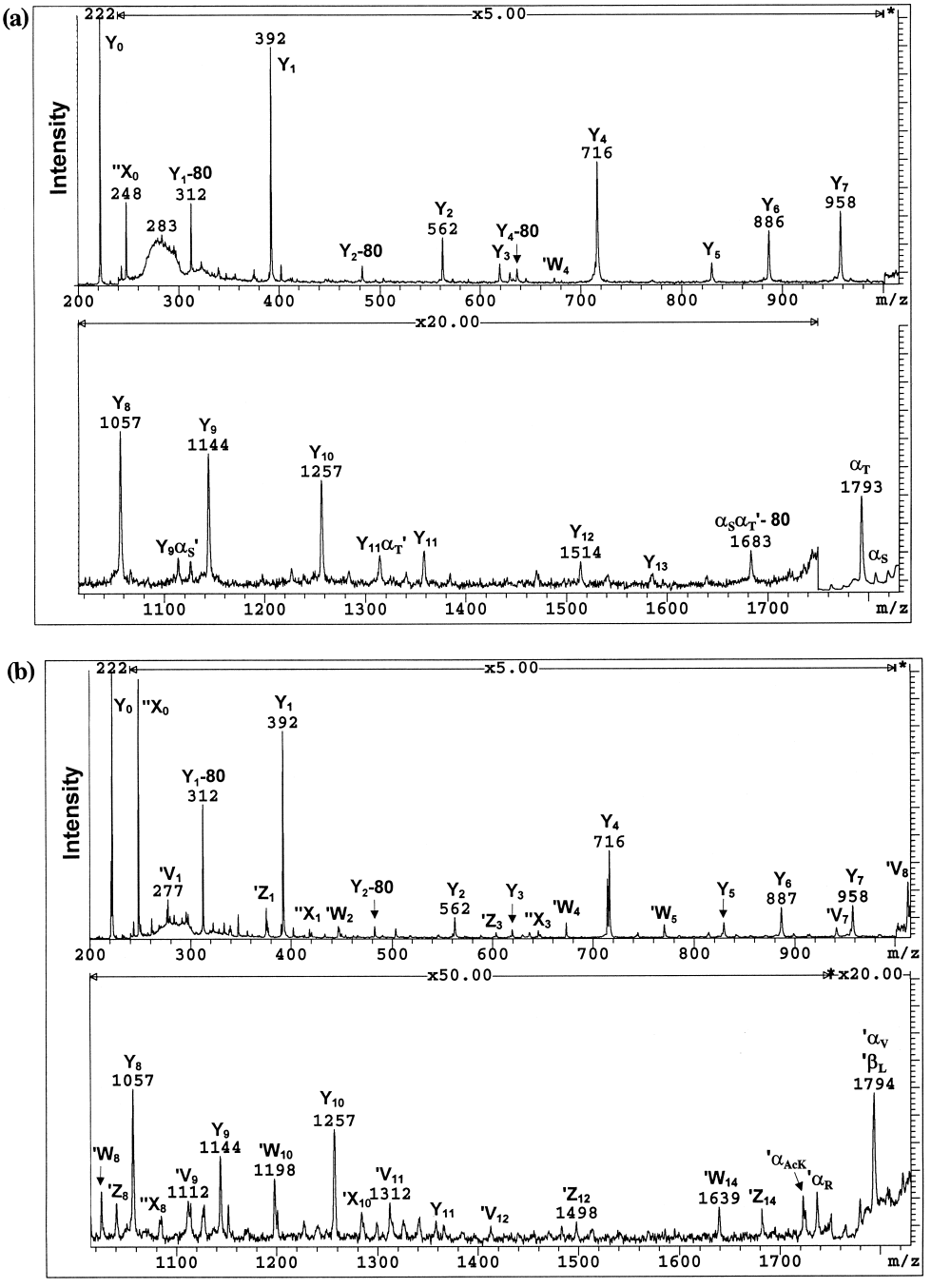


Figure 1. Nano-ES/CID spectra of the $[M - H]^-$ ion of the acetylated and naphthalenesulfonated peptide Syntide 2 (Ac-PLARTLSVAGLPGKK-ansa) obtained using (**a**) methane and (**b**) xenon as the collision gas. The two spectra provide complementary information for the deduction of the amino acid sequence.

Shown in Figure 1a is the CID spectrum of the acetylated and naphthalenesulfonated peptide Syntide 2 (Ac-PLARTLSVAGLPGKK-ansa) obtained with methane as the collision gas. The dominating Y fragment ion series makes interpretation of spectrum straightforward.

The following features are characteristic of spectra obtained with *xenon* as collision gas:

- The most intense peaks usually correspond to Y, 'V, and 'W fragment ions.
- 'V fragment ions are observed for all amino acid residues except proline and glycine which cannot give this fragment.
- 'W fragment ions are generally observed for amino acid residues with the relevant side chains, except tryptophan, histidine, tyrosine, and phenylalanine,

i.e., the aromatic amino acid residues. This is analogous to earlier observations on protonated peptides made by Biemann and co-workers [34].

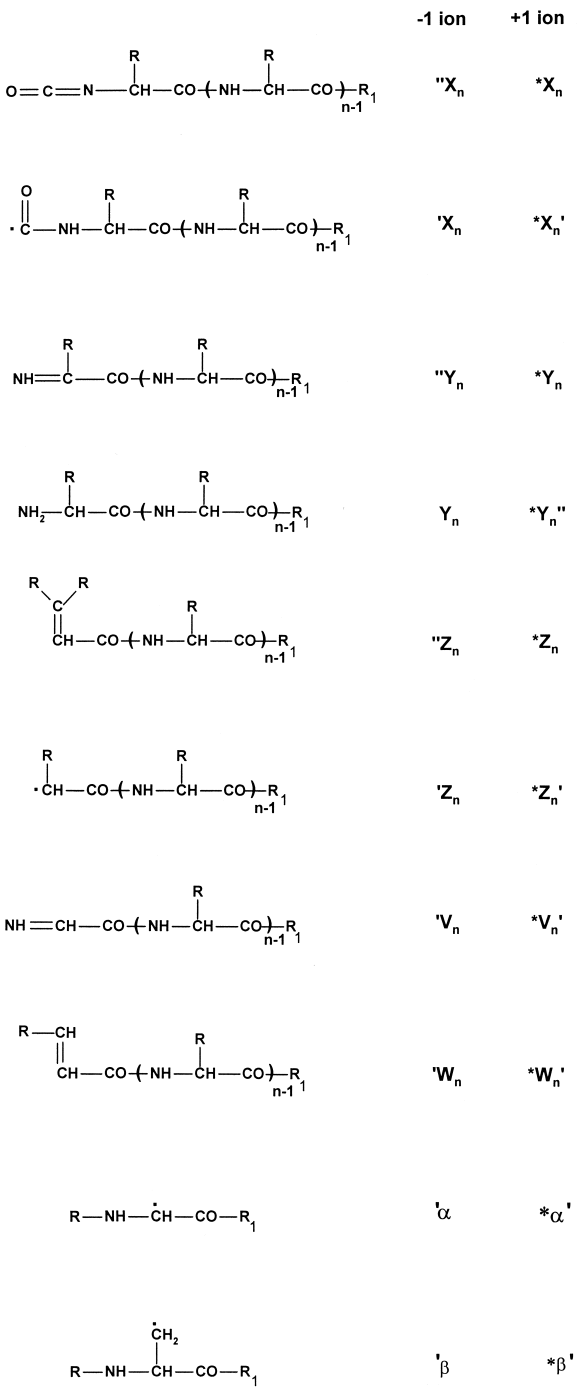
- The X-type fragment ions appear as $''X_n$ or $'X_n$, or as a $''X_n/'X_n$ doublet. The Z-type fragment ions appear as $'Z_n$ or as a $''Z_n/'Z_n$ doublet. All these fragment ions are frequently observed and are usually of medium intensity.
- The $'\alpha x$ fragment ions, corresponding to a side-chain neutral loss, are usually observed for all amino acid residues (including tryptophan) except for glycine, proline, serine, and threonine (discussed below) and are in most cases considerably more abundant than the backbone fragment ions in the high *m/z* region of the spectra.
- $'\beta x$ fragment ions are sometimes present and are generally of lower abundance than the $'\alpha x$ fragment ions.
- αs, αt, and combined losses of the side chains of serine and threonine, giving for example $\alpha s\alpha t'$ fragment ions, are generally observed in spectra of peptides containing serine and/or threonine.
- Combinations of the αs and αt side-chain losses with other fragmentation are also seen. The most common combination is with Y fragment ions giving $Y_n\alpha s'$ or $Y_n\alpha t'$ fragment ions (see Table 2).

Shown in Figure 1b is the CID spectrum of the acetylated and naphthalenesulfonated peptide Syntide 2 (Ac-PLARTLSVAGLPGKK-ansa) obtained with xenon as the collision gas.

Mechanisms of Fragmentation

Derivatization of the carboxylate group of biomolecules with aminosulfonic acids has been employed previously in this laboratory to enhance the CRF of deprotonated molecules upon high energy CID [23–26, 36–38]. Aromatic sulfonate derivatizing groups have been found to be particularly favorable in promoting CRF reactions on account of their ability to strongly bind negative charge. Furthermore, the planar nature of the aromatic group isolates the charge site from the remainder of the ion, thus minimizing the possibility of charge mediated processes. It has shown that at a collision energy of 400 eV CRF reactions dominate the CID spectra of deprotonated aromatic sulfonates [37].

Although numerous studies have been made of the CID of peptides derivatized at the N-terminus with a positively charged group [14–17, 22], far fewer studies have been made when the C-terminus is derivatized in this way [16, 18]. Watson et al. derivatized synthetic peptides at the C-terminus with an aminoethyl-triphenylphosphonium reagent [16, 18]. The ethyl-triphenylphosphonium (ethyl-TPP) derivatized peptides thus formed were subjected to high-energy CID. The C-terminally derivatized peptides were found to give spectra containing prominent Y-type, and also X-, Z-, V-, and W-type fragment ions [16]. CRF mechanisms were proposed to account for the formation of these ions and their suggested structures are given in Scheme **2**. When using the Roepstorff and Fohlman [30] or the alternative Biemann [39] nomenclature a complication arises for peptide ions carrying a positively charged quaternary phosphonium (or ammonium) group. Such peptides in the singly charged form are not protonated.



Scheme 2. Structures of C-terminal fragment ions formed by CRF mechanisms. In the present study $R_1 = NHC_{10}H_6SO_3^-$, in [16] $R_1 = NHC_2H_4P^+(C_6H_5)_3$.

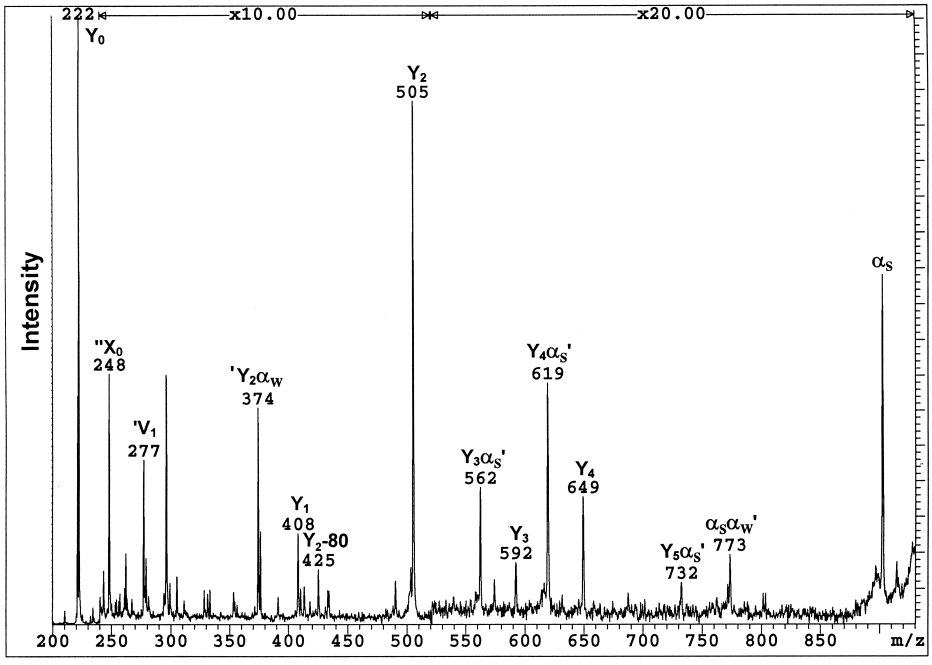


Figure 2. Nano-ES/CID spectrum of the $[M - H]^-$ ion of a acetylated and naphthalenesulfonated peptide obtained from proteolytic cleavage of aldehyde dehydrogenase, using methane as the collision gas, m/z = 932.4.

If the derivatizing group is localized at the C-terminus, homolytic cleavage of, for example, a NH–CHR bond without hydrogen transfer would lead formally to a Z_n fragment according to the Roepstorff and Fohlman nomenclature and a z_n fragment according to the Biemann nomenclature. However, to take account of the positively charged derivatizing group Watson and co-workers [18] classified the formal z_n fragment as $*z_n$ + 1. This is reasonable as a z_n + 1 ion in the Biemann nomenclature corresponds to a distonic radical cation protonated on a residue towards the C-terminal. Watson's use of the asterisk indicates the charge is carried by the derivatizing group as opposed to a proton. Similarly the $*z_n$ + 1 ion can be described by $*Z'_n$ in the Roepstorff and Fohlman nomenclature. Watson and co-workers have subsequently used the modified Biemann nomenclature in recent reports of the fragmentation of peptides derivatized with quaternary phosphonium groups and analyzed by ES MS/MS and MALDI PSD [17–20].

In the high-energy CID study of peptides derivatized at the C-terminus with an ethyl-TPP group Watson et al. [16] observed abundant *Y (*y − 2) rather than *Y″ (*y) fragment ions. They proposed a CRF mechanism to account for the formation of the *Y ion in which a H is transferred from the α-carbon immediately C-terminal to the fragmenting bond, onto the carbonyl group N-terminal to the breaking bond. A CRF mechanism was also proposed to account for the formation of the *X (*x) ion involving H transfer from the nitrogen C-terminal to the fragmenting bond onto the α-carbon N-terminal to the cleaving bond. Although Watson et al. [16] did not propose structures or mechanisms for the formation of the other peptide fragment ions in their spectra, i.e., *Z′ (*z + 1), *V′ (*v), and *W′ (*w), these ions are believed to be formed by CRF mechanisms [18] and have the structures given in Scheme **2**.

Previous studies on the fragmentation of deprotonated aromatic sulfonic acid derivatives [23–26, 36–38] suggest that the reactions occur via CRF mechanisms. It is highly probable that this is true for the deprotonated molecules investigated here. As in the study reported by Watson et al. [16] Y-, X-, Z-, V-, and W-type fragment ions were observed in the spectra presented here in Figures 1–4 and described in Tables 1 and 2. The proposed structures of these fragment ions are given in Scheme **2**. It can be seen that with the exception of the derivatizing group the ions proposed to be formed by fragmentation of the positively charged and the negatively charged C-terminally derivatized peptides are identical. One notable difference in the fragmentation of positive- and negative-charged derivatized peptides concerns the Y-type fragment ions. With the positively charged derivative, terminally unsaturated *Y (*y − 2) ions were predominately formed, while with the negatively charged derivative terminally unsaturated Y-type ions (″Y) were only formed in abundance when cleavage was of the amide bond N-terminal to a proline residue. This observation has also been made for the fragmentation of underivatized peptides [32]. The terminally saturated Y ions observed in the CID spectra of the negatively charged derivative are also believed to be formed via a CRF mechanism [32].

The other fragment ions observed in the present

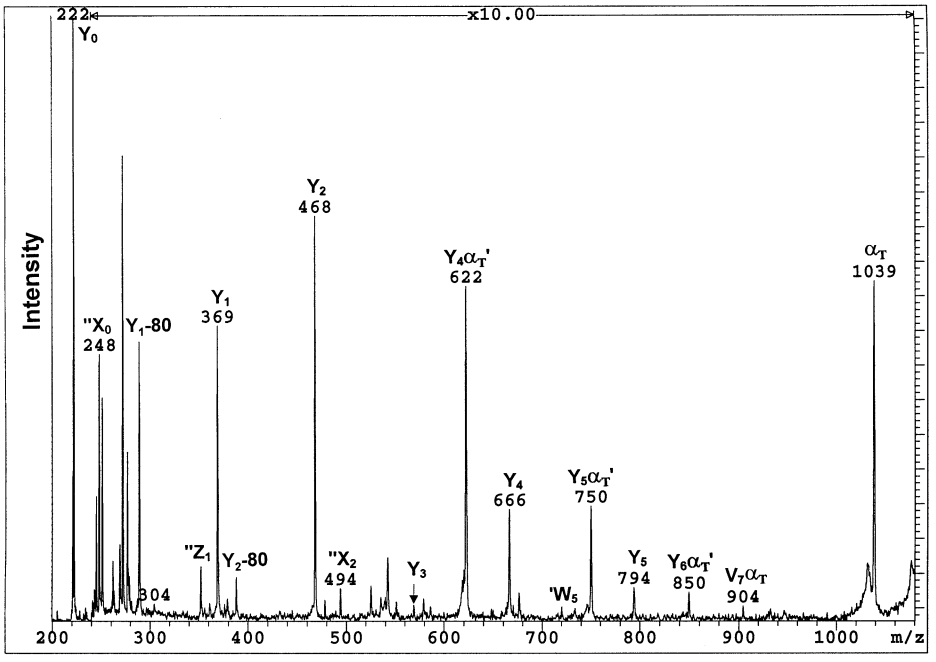


Figure 3. Nano-ES/CID spectrum of the $[M - H]^-$ ion of a acetylated and naphthalenesulfonated peptide obtained from proteolytic cleavage of aldehyde dehydrogenase, using methane as the collision gas, m/z = 1082.5.

study (Figures 1–4), i.e., $'\alpha$, $'\beta$, αs and αT are formed as a result of side-chain losses, these ions are also expected to be formed by CRF, and their proposed structures are given in Scheme **2**.

The mechanisms believed to be responsible for the fragmentation of naphthalenesulfonated peptides are dissimilar to the charge directed mechanisms proposed by Waugh and Bowie [35] to account for fragment ions present in the spectra of underivatized deprotonated peptide molecules. The absence in underivatized peptides of a group which strongly binds negative charge discourages CRF from occurring.

Synthetic Peptides Containing Asp and Glu

The synthetic peptides used in the present study did not contain aspartic or glutamic acid residues. When these are present, the carboxyl group of the side chain is also derivatized. The EDC-activated carboxyl group may also react with an NH group of the peptide backbone to form a 5- or 6-membered ring. The structures of these side products and their utility in sequence determinations are presently being studied. Their formation may lower the sensitivity, but do not invalidate the use of the derivatization method for sequencing purposes. If the acidic residue is close to the C-terminal ansa, the appearance of the spectrum will be similar to that of a peptide derivatised only at the C-terminus except that the masses of glutamic and/or aspartic acid residues will be increased by 205 Da due to the addition of ansa. If the acidic residue is in the middle of the peptide or close to the N-terminus, the spectrum will exhibit N-terminal fragment ions arising from cleavages at the amino acid residues on the C-terminal side of the derivatized residue, i.e., the spectrum will contain both N- and C-terminal fragment ions. This complicates spectrum interpretation. In the sequence analysis of proteins, exclusive C-terminal derivatization of proteolytic peptides can be achieved through the use of a Glu/Asp-specific protease.

Peptides from Proteolysis of ALDH

The use of the derivatization method for the sequence analysis of noncharacterized peptides was evaluated with peptides obtained by proteolysis of cytosolic ALDH from hamster liver. Peptides in the digests were fractionated by HPLC and positive-ion nano-ES spectra were recorded. Eight fractions were selected for derivatization, negative-ion mass spectra recorded and peaks corresponding to acetylated and aminonaphthalenesulfonated peptides were identified. The $[M - H]^-$ ions of the derivatized peptides were subjected to CID using methane and/or xenon as the collision gas. The spectra were interpreted using the guidelines given above. Complete or partial sequence information was obtained. Any lack of sequence ions was due to a low precursor ion current, and in such cases the N-terminal part of the peptide was undefined. CID spectra from three peptides are shown in Figures 2–4.

The amino acid sequence information for five previously noncharacterized peptides obtained from the pro-

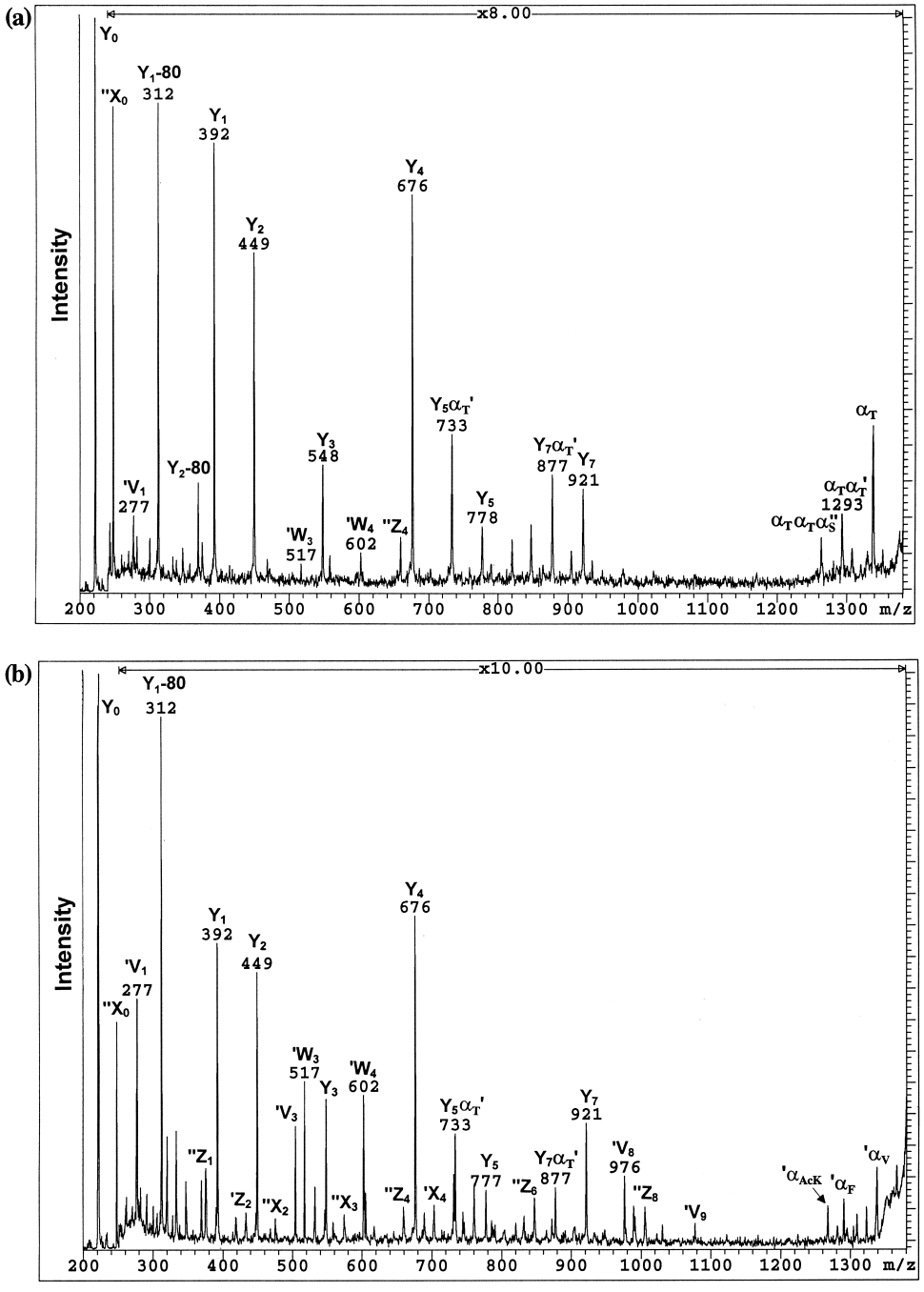


Figure 4. Nano-ES/CID spectra of the $[M - H]^-$ ion of a acetylated and naphthalenesulfonated peptide obtained from proteolytic cleavage of aldehyde dehydrogenase, using (**a**) methane (**b**) xenon as the collision gas, m/z = 1381.6.

teolysis of hamster liver ALDH is presented in Table 3. As can be seen complementary data are obtained with xenon and methane as the collision gases. For example, when considering both sets of data, the deprotonated molecule at m/z 932.4 (see Table 3) can only have the amino acid sequence QIGSPW. Although in three cases complete amino acid sequence information was not provided, a stretch of amino acids of sufficient length to apply to a data base algorithm was obtained. In this example, the protein was not in the database. However, it is necessary to confirm that the proteolytic peptides analyzed are not from contaminating proteins.

In the present paper we have illustrated the value of C-terminal derivatisation of peptides for their sequence analysis. The studies performed on noncharacterized peptides show that this method is viable for de novo sequencing. Although the derivatization method is time consuming and can result in unwanted side products, it should be regarded as a complementary aid in protein sequence analysis by mass spectrometry.

Table 3. Amino acid sequence information deduced from the CID spectra of previously noncharacterized peptides obtained from proteolytic cleavages of cytosolic hamster liver aldehyde dehydrogenase and derivatized with ansa. The interpretation was in some cases confirmed by Edman sequencing

$[M - H]^-$ *m/z*	Amino acid sequence information in CID spectra	
	Collision gas:methane	Collision gas:xenon
932.4	X[a]IGSPW	(QIGS)[b]PW
1082.5	FVQPTVF	(FV)[b]QPTVF
1136.5	−[c](L/I)[d]K[e](L/I)[d]QY	−[c]IQY
1381.6	−[c]TGSTQVGK[e]	(VAF)[b]TGSTQVGK[e]
1956.9	Not analyzed	[c]AAGVFTK[e]
2111.9	Not analyzed	−[c]ANLK[e]

[a]X can be Q or AG.
[b]Information as to the identity of the residues in parentheses can be deduced from the spectra, but their relative positions cannot be established.
[c]The sequence of the N-terminal part of the peptide cannot be deduced from the spectra.
[d]Leucine and isoleucine cannot be distinguished.
[e]All lysine residues are *N*-acetylated.

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